EXPRESSION VECTOR FOR HIRUDIN AND TRANSFORMED CELLS AND TRANSGENIC ANIMALS CONTAINING SAID VECTOR

TECHNICAL FIELD

[0001] The present invention relates to an expression vector, a transformed cell containing the same. Also, a transgenic non-human mammal whose genome comprises a DNA construct comprising in operable association promoter specifically expressing gene in a mammary gland cell or tissue and a nucleic acid encoding hirudin and cells isolated from the transgenic non-human mammal are provided.

BACKGROUND ART

[0002] Hirudin is a polypeptide with anti-thrombotic activity consisting of 65 to 66 amino acids, which is isolated from the salivary glands of *Hirudo medicinalis*. Three hirudin variants, HV1, HV2 and HV3 are known as natural hirudins, which have some differences in their numbers of amino acids and protein structures. Hirudin has a high specificity to thrombin, thereby it can combine with thrombin to inhibit the coagulation ability of thrombin. Therefore, hirudin can be used in the treatment of anticoagulation.

[0003] In early times hirudin was obtained by purifying and isolating it from the salivary glands of *Hirudo medicinalis*. It is difficult to provide large amounts of hirudin for medical use. The production of hirudin by gene recombinant technology started from the discovery of the amino acid sequence and the protein structure of hirudin (Dodt *et al.*, FEBS Lett. 165: 180-184, 1984). EP 158,564, EP 168,342, EP 171,024, EP 412,526, EP 687,731 and USP 5,824,505 disclosed the production of recombinant hirudin using prokaryotic system, such as *Escherichia coli*. The hirudin produced by *E. coli* is secreted to the periplasmic space. To recover the hirudin, it is necessary to disrupt the bacterial cells. Thus the yield of the hirudin is low. In addition, since the prokaryotic system lacks post-translational modification, the biological activity of the resulting hirudin is also poor.

[0004] In the eukaryotic expression system, yeast is customarily used as the host cell. However, the C-terminal of the resulting hirudin will be degraded by carboxypeptidase. USP 5,866,399 disclosed a carboxypeptidase-deficient yeast to produce hirudin. However, the yield produced by such a yeast method is low.

DISCLOSURE OF INVENTION

[0005] One object of the invention is to provide an expression vector for expressing a hirudin comprising a mammalian origin of replication, in operable association promoter specifically expressing gene in a mammary gland cell or tissue and a nucleic acid encoding hirudin.

[0006] Another object of the invention is to provide a transformed mammary gland cell which comprises the expression vector of the invention.

[0007] One further object of the invention is to provide a non-human transgenic mammal whose genome comprises a DNA construct which prepared from above expression vector, comprising in operable association promoter specifically expressing gene in a mammary gland cell or tissue and a nucleic acid encoding hirudin.

[0008] Another further object of the invention is to provide the mammalian cells which are isolated from above transgenic non-human mammal whose genome comprises a DNA construct which prepared from above expression vector.

[0009] Another further object of the invention is to provide a polynucleotide for amplifying the gene of the hirudin, which is selected from the group consisting of SEQ ID Nos.1, 2, 3 and 4.

[0010] Another further object of the invention is to provide a primer for amplifying the gene of the hirudin, which is selected from the group consisting of SEQ ID Nos.5, 6, 7 and 9.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] Fig. 1 shows the polynucleotide chain sequences of Hi-AF, Hi-AR, Hi-BF and Hi-BR.

[0012] Fig. 2 shows the nucleotide sequence of primer for the PCR of Hi-PCR-AF, Hi-PCR-AR, Hi-PCR-BF and Hi-PCR-BR.

[0013] Fig. 3 shows the scheme for the synthesis of the fragment of hirudin gene.

[0014] Fig. 4 shows the nucleotide sequence of hirudin gene and the amino acid thereof.

[0015] Fig. 5 shows the construction of the expression vector for specifically expressing in the mammary gland cell and tissue.

[0016] Fig. 6 shows a plot of plasma coagulation of the gene-transformed mammary gland cell or the homogenization solution of the gene-transformed mammary gland tissue.

[0017] Fig. 7 shows a plot of plasma coagulation of the gene-transformed mammary gland cell or the culture solution of the gene-transformed mammary gland tissue.

[0018] Fig. 8 shows analysis of αLA-hirudin transgenic mouse (A) and pig (B) by polymerase chain reaction. "+", "-", "Tg" and "W" presented positive, negative, transgenic animals and water, respectively.

BEST MODE FOR CARRYING OUT THE INVENTION

[0019] The present invention features an expression vector for expressing a hirudin in a mammary gland cell or tissue. Comprising in operable association promoter specifically expressing gene in a mammary gland cell or tissue and a nucleic acid encoding hirudin. The present invention also provides a transformed mammary gland cell. An transgenic mammal whose genome contains a DNA construct prepared from such expression vector and cells isolated from such transgenic non-human mammal are also disclosed.

Definitions

[0020] The term "hirudin", as used herein, refers to any one of the forms of natural or synthetic hirudin, that is to say, a product having the same activity *in vivo* as hirudin, which will sometimes be referred to as a hirudin analog.

[0021] The term "expression vector", as used herein, refers to a vector capable of directing the expression of a gene to which it is operatively linked. In general, an expression vector of utility in recombinant DNA techniques is often in the form of a "plasmid" which refers generally to a circular double stranded DNA loop which, in its vector form is not bound to the chromosome.

[0022] The term "host cell", as used herein, refers to a cell of a host which can be infected with a vector, such as a plasmid.

[0023] The "non-human mammal" of the invention comprises any non-human mammal whose genome contains an expression vector as described herein. Such non-human mammals include, but are not limited to, rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.

[0024] The term "transgene" as used herein refers to a foreign gene that is placed into an organism by introducing the foreign gene into an embryonic stem (ES) cells, newly fertilized eggs or early embryos.

[0025] The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor. The polypeptide can be encoded by a full-length coding sequence or by any portion of the coding sequence, so long as the desired enzymatic activity is retained.

[0026] The terms "in operable association", "in operable order" and "operably linked", as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The terms also refer to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

[0027] The terms "promoter element" or "promoter", as used herein refer to a DNA sequence that is located at the 5' end of (i.e., precedes), a gene in a DNA polymer and provides a site for initiation of the transcription of the gene into mRNA.

Primers, Expression Vector and Host System

[0028] One object of the invention is to provide an expression vector for expressing a hirudin comprising a mammalian origin of replication, in operable association promoter specifically expressing gene in a mammary gland cell or tissue and a nucleic acid encoding hirudin.

[0029] According to the invention, the nucleic acid encoding hirudin is operably linked to a promoter specifically expressing gene in a mammary gland cell or tissue. According to the invention, the expression vector has the mammalian origin of replication.

[0030] According to the invention, the vector can be obtained through the synthesis and cloning of the hirudin gene. The hirudin gene sequence has been disclosed in GenBank, (Accession number M26726).

[0031] It is difficult to obtain the *Hirudo medicinalis* so that the hirudin cannot be produce in large mass by using the construct of gene library or reverse-transcriptase polymerase chain reaction. According to the invention, the synthesis and cloning of the gene of the hirudin are via chemical synthesis method. Four polynucleotides are designed based on the sequence of the hirudin gene as disclosed in the GeneBank (see SEQ ID Nos.1, 2, 3 and 4). According to the invention, new primers are designed based on the 5' and 3' end of the SEQ ID Nos.1, 2, 3 or 4 to conduct the polymerase chain reaction. Preferably, the primers are selected from the group consisting of SEQ ID Nos. 5, 6, 7 and 8.

[0032] The gene fragment of hirudin can be amplified through PCR using the above-mentioned

polynucleotides chains and primers. The resulting fragment is cut by appropriate restriction enzymes and then cloned to an expression vector containing a promoter specifically expressing gene in a mammary gland cell or tissue. Preferably, the promoter is isolated from human, pig, cattle, horse, goat, camel, sheep or rodent. More preferably, the promoter is isolated from the mammary gland cell or tissue of human, pig, cattle, horse, goat, camel, sheep or rodent. More preferably, said promoter is selected from the group consisting of casein gene, whey acid protein gene, lactoalbumin gene and lactoglobulin gene. Most preferably, said lactoalbumin promoter is α-lactoalbumin promoter.

[0033] Another object of the invention is to provide a transformed mammary gland cell which comprises an expression vector for expressing a hirudin in mammary gland cell or tissue, comprising in operable association a promoter specifically expressing gene in a mammary gland cell or tissue and a nucleic acid encoding hirudin. Preferably, the promoter is isolated from human, pig, cattle, horse, goat, camel, sheep or rodent. More preferably, the promoter is isolated from the mammary gland cell or tissue of human, pig, cattle, horse, goat, camel, sheep or rodent. More preferably, said promoter is selected from the group consisting of casein gene, whey acid protein gene, lactoalbumin gene and lactoglobulin gene. Most preferably, said lactoalbumin promoter is α-lactoalbumin promoter. According to the invention, the mammary gland cell is from various mammals. Preferably, the mammary gland cell is from human, pig, goat, sheep, camel or rodent.

Transgenic Animal

[0034] One further object of the invention is to provide a non-human transgenic mammal whose genome comprises a DNA construct which is prepared from expression vector for expressing hirudin in mammary gland cell or tissue comprising in operable association promoter specifically expressing gene in a mammary gland cell or tissue and a nucleic acid encoding hirudin. Preferably, the promoter is isolated from human, pig, cattle, horse, goat, camel, sheep or rodent. More preferably, the promoter is isolated from the mammary gland cell or tissue of human, pig, cattle, horse, goat, camel, sheep or rodent. More preferably, said promoter is selected from the group consisting of casein gene, whey acid protein gene, lactoalbumin gene and lactoglobulin gene. Most preferably, said lactoalbumin promoter is α-lactoalbumin promoter.

[0035] Recent advances in molecular genetics have provided powerful tools for the generation

of novel transgenic animals for the study of human disease or the production of useful substances. A gene of interest can be introduced into a mouse by standard transgenic methods. For example, embryonal cells at various developmental stages can be used to introduce transgenes for the production of transgenic animals. Different methods are used depending on the stage of development of the embryonal cell. The zygote is the best target for micro-injection. The use of zygotes as a target for gene transfer has a major advantage, in that, in most cases the injected DNA will be incorporated into the host genome before the first cleavage. As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. Micro-injection of zygotes is the preferred method for incorporating transgenes in practicing the invention.

[0036] Another object of the invention is to provide a mammalian cell, which is isolated from transgenic non-human mammal of the invention. Preferably, the mammalian cell is a mammary gland cell.

Production of hirudin

[0037] Another further object of the invention is to provide a method of producing the hirudin, which culturing the transformed mammary cells with the expression vector of the invention under appropriate cultivation conditions and then recovering the hirudin expressed by said cells. According to the invention, the cultivation conditions of the mammary cells and the steps and conditions of the recovering the hirudin are known in the art.

[0038] On the other hand, the hirudin also can be produced by the transgenic animals of the invention.

Utility

[0039] The present invention provides an expression vector for expressing hirudin, the cells containing the vector which permit the expression of hirudin, as well as the a transgenic mammal and isolated cells from such mammal whose genome containing a DNA construct prepared from above expression vector so that the hirudin can be produced by the transgenic animal or isolated mammalian cells.

[0040] The transgenic mammal permits the production of hirudin in large quantities and are easily recoverable. It is therefore advantageous that the recombinant hirudin is produced in the mammary gland of transgenic animals and excreted in their milk. It is indeed a biological fluid which can be easily collected, having a relatively limited complexity and a low proteolytic

activity; in addition, the processes of maturation of the recombinant proteins will be probably ensured, (glycosylation, phosphorylation, cleavage and the like). Given the above, the hirudin can be largely produced from the transgenic animal of the invention and has biological activity.

[0041] The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1: Artificial Synthesized Hirudin Gene

1.1: Synthesis of Polynucleotide Chains of Hirudin Gene

[0042] Polynucleotide chains were designed and synthesized based on the hirudin nucleotide sequence of the accession number M26726 in the databank GenBank. As shown in Figure 1, the synthesized polynucleotide chains are Seq1 (Hi-AF), Seq2 (Hi-AR), Seq3 (Hi-BF) and Seq4 (Hi-BR), wherein Hi-AF and Hi-AR are complementary sequences and Hi-BF and Hi-BR are complementary sequences. Moreover, PCR primers are designed according to the 5'-terminal sequences of both the forward and reverse strands of Hi-AF, Hi-AR, Hi-BF and Hi-Br. As shown in Figure 2, the primers are Seq5 (Hi-PCR-AF), Seq6 (Hi-PCR-AR), Seq7 (Hi-PCR-BF) and Seq8 (Hi-PCR-BR), respectively.

1.2: Synthesis of Hirudin Gene Sequences

1.2.1: Amplification of Hirudin Gene Fragments by PCR

[0043] PCR was carried out respectively using the polynucleotide chains Hi-AF and Hi-AR as the templates and Hi-PCR-AF and Hi-PCR-AR as the primer pair and using the polynucleotide chains, Hi-BF and Hi-BR as the templates and Hi-PCR-BF and Hi-PCR-BR as the primer pair to amplify the complementary sequences of the hirudin gene (designated as Hi-A and Hi-B, respectively). Ten-time PCR buffer (10 μl, comprising 15 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100, 0.1% gelatin and 100 mM Tris-HCl, pH 7.9), dATP, dCTP, dTTP and dGTP (200 μM each), and the above primer pairs (0.2 μM each) were added into the above template DNAs (1 ng each). *Tth* polymerase (0.5 U, purchased from Promega Co., USA) was the final ingredient. The total reaction volume is 100 μl.

[0044] The above mixtures were heated at 94°C for 5 minutes before entering the PCR cycles. The reaction conditions are 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 45 seconds. After a total of 40 cycles, the mixtures were subjected to 72°C for 3 minutes to complete the DNA extension.

1.2.2: Annealing of Hirudin Gene sequences

[0045] As shown in Figure 3, a cutting site for the restriction enzyme *Xba*I was designed at both the 3'-terminal of Hi-A and 5'-terminal of Hi-B. The amplified products of Hi-A and Hi-B obtained from Step 1.2.1 were purified and recovered with a PCR purification kit, (PCR Clean Up-M, purchased from Viogene) and then cut by the restriction enzyme, *Xba*I. The cut DNA fragments of Hi-A and Hi-B were electrophorized and recovered from 2% agarose gel by Gel Extraction Kit (purchased from Viogene). The two fragments were ligated to obtain a DNA fragment containing the complete hirudin gene sequence, which was designated as, Hi. Fig. 4 (A) shows the obtained hirudin gene sequence (SEQ ID NO:9); Fig. 4 (B) shows the obtained hirudin amino acid sequence (SEQ ID NO:10), wherein amino acid 33 is different from that of HV1 hirudin sequence, (aspartic acid to arginine). The above enzyme cutting reaction and ligation both employ known standard methods, (see Molecular Cloning-A Laboratory Manual; Cold Spring Harbor, 1989).

Example 2: Construction of Expression Vector Specifically expressing Hirudin in Mammary Gland Cells and Mammary Gland Tissues

2.1: Construction of Expression Vector pE-Hi

[0046] As shown in Figure 5, cutting sites for the restriction enzymes *Bam*HI and *Not*I were designed at both the 5'-terminal of Hi-A and 3'-terminal of Hi-B in Step 1.1. The DNA fragment, Hi containing the complete hirudin gene sequence obtained from Step 1.2.2 were cut by the restriction enzymes *Bam*HI and *Not*I, DNA fragment sized 215 bp was electrophorized and recovered from 2% agarose gel by Gel Extraction Kit. Ligation was performed with the above purified DNA fragment and the *Bam*HI/*Not*I cut vector pEGFP-1 (pEGFP-N1 without the EGFP sequence fragment) to obtain the expression vector pE-Hi (3.6 kb). pE-Hi was transformed into *E. coli* NM522 competent cells and the anti-ampicillin transformants were selected. The above transformation employs a known method, (Molecular Cloning-A Laboratory Manual; Cold Spring Harbor, 1989).

2.2: Construction of Hirudin-Expressing Vector

2.2.1: Purification of Plasmid DNA

[0047] Bacto-trypton (10 g), yeast extract (5 g) and NaCl (10 g) were dissolved in de-ionized water (1 L). After adjusting the pH to 7.5, the solution was autoclaved. After cooling Luria-Bertani (LB) medium was obtained.

[0048] Ampicillin (50 mg/mL, 10 μl), was added into the obtained LB medium (10 mL), to

a final concentration of 50 μg/mL and LB/Amp medium was obtained. The anti-ampicillin transformants selected in Step 2.1 were inoculated into the LB/Amp medium. After cultured with agitation at 37°C for 24 hours, the bacteria were collected and the pE-Hi vector DNA was purified by Plasmid DNA Mini-M™ Extraction System(purchased from Viogene).

2.2.2: Construction of Expression Vector pE-αLA-Hi

[0049] As shown in Figure 5, the expression vector pE-Hi was cut by the restriction enzymes *Bam*HI and *Xho*I, and the cut 3.6-kb DNA fragment was electrophorized and recovered with 1% agarose gel. Ligation was performed with said DNA fragment and a 1.9-kb DNA fragment containing the promoter of α-lactoalbumin recovered from *Bam*HI/ *Xho*I cut pαLA-hFIX (S. P. Lin, Construction and Expression of hybrid gene contained the promoter of α-lactalbumin and the cDNA of human blood clotting factor IX, Master's Thesis of Department of Animal Science of National Taiwan University, 1996), and an expression vector specifically expressing hirudin in mammary gland cells and mammary gland tissues was obtained and named, pE-αLA-Hi.

Example 3: Detection of Expression of Hirudin-expressing Vector in Mammary Gland Cell Line

3.1: Culture of Mammary Gland Cell Line

[0050] The murine mammary gland epidermal cell line NMuMG (CCRC 60087), was purchased from the cell bank of the National Health Research Institute (Taipei, Taiwan, ROC). NMuMG cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 45 mg/mL glucose and 10% fetal bovine serum (FBS), at 37°C 5% CO_2. NMuMG cells were grown in the above culture conditions have an appearance of a single polygon without tentacles, and do not have the function of a differentiated mammary gland cell, i.e. transfer or secretion of large amounts of proteins out of the cell. However if 5 μg/mL insulin , 5 μg/mL prolactin and 1 μg/mL dexamethasone were added to the culture medium and a layer of Matrixgel (50ml/cm^2, purchased from Sigma), was coated on the bottom of the petri-dish, after culturing the NMuMG cells for 24 hours, obvious cell colonies would appear. Each colony consists of tens of thousands of aggregating cells and takes the form of a hollow hemisphere, which is similar to the appearance of a lactating cell cluster of the mammary gland, *in vivo*.

3.2: Gene Transformation of Mammary Gland Cells

[0051] The pE-αLA-Hi vector DNA (5 μg/50 μL) was uniformly mixed with liposomes (100

μL, SuperFact, purchased from QIAGENE), and then serum-free DMEM medium (850 μL) was added to become a "DNA-liposome-medium solution", which can be used for the gene transformation of cells. NMuMG cells cultured to a density of 60-80% on Matrixgel without addition of hormones (insulin, prolactin and dexamethasone) were rinsed with 1-time phosphate-buffered saline (PBS, pH 7.4), for 3 times. The above DNA-liposome-medium solution was added therein and the cells were incubated in an incubator at 37°C, 5% CO_2 for 1 hour. Then DMEM medium (4 mL), containing 20% FBS was added. After continuously culturing the cells for 24 hours, the solution containing DNA and liposomes was removed and DMEM medium containing 10% FBS was added. Geneticin (final concentration was 500 μg/mL, also called G418, purchased from Sigma) was added at the same time to select successfully gene-transformed cells. The culture medium was replaced irregularly thereafter and after culturing for 2 successive generations, NMuMG cell lines with the pE-αLA-Hi stably existing and expressed in the presence of G418 (pE-αLA-Hi/NMuMG), could be obtained.

Example 4: Determination of the Biological Activity of Hirudin

4.1: Expression of Hirudin by Mammary Gland Cell Line

[0052] The pE-αLA-Hi transformed mammary gland cell line (pE-αLA-Hi/NMuMG) obtained from Step 3.2 was cultured in the mammary gland cell culture medium containing hormones (insulin, prolactin and dexamethasone) and 500μg/mL geneticin in a petri dish coated with Matrixgel, (as described in Step 3.1), at 37°C and 5% CO_2. When cell colonies showed an appearance of a hollow hemisphere like the mammary gland follicle, the cell culture medium was moved into a clean test tube. Mammary gland cells were separated from the petri dish with 0.25% trypsin solution. After centrifugation at 1,000 rpm for 5 minutes, the supernatant was removed and cells were collected. The cells were resuspended in 1 mL hypertension solution (25% sucrose, 1 mL EDTA and Tris-HCl, pH 7.5) and reacted in room temperature for 15 mins. Then the cells were broken with a sonicator and centrifuged at 6,000 rpm for 10 minutes to remove cell debris. The obtained supernatant is the hirudin-containing homogenized cell solution.

[0053] Determination of the anti-coagulation biological activity of hirudin was carried out independently with the homogenized mammary gland cell solution and the cell culture described above. The total amount of protein in the homogenized cell solution was determined to be the basis and unit of addition of the homogenized solution in the anti-coagulation

biological activity determination.

4.2: Determination of Anti-coagulation Biological Activity

[0054] Bovine thrombin (purchased from Sigma), with a concentration of 0.2 pmole was prepared with analysis buffer, (0.12 M NaCl, 0.01 M sodium phosphate, 0.01% NaN_3 and 0.1% bovine serum albumin, pH 7.4). Various concentrations (0.04, 0.08, 0.16, 0.32, 0.64, 1.28 and 2.56 pmole) of market available natural hirudin (purchased from Sigma), various amounts (0.03, 0.06, 0.43, 0.25, 0.5, 1 and 2 mg total protein) of homogenized gene-transformed mammary gland cell solution or various volumes (0.15, 0.31, 0.62, 1.25, 2.5, 5 and 10 μL), of gene-transformed mammary gland cell culture medium were independently mixed with 50 μL bovine thrombin described above. After reacting at 24°C for 1 minute, 100 μL 10-time analysis-buffer-diluted human serum was added, mixed and allowed to react for 20 seconds. After 15 minutes the absorbance at 405 nm (A_{405}), of the reaction mixture was determined. It showed the lower the A_{405} reading value was, the higher the anti-coagulation level was.

[0055] As shown in Figure 6 and 7, when the concentration of the natural hirudin is 0.16 pmole, there is almost no coagulation; a similar no-coagulation situation happens when the amounts of the homogenized gene-transformed mammary gland cell solution and the gene-transformed mammary gland cell culture are 0.25 mg and 10 μL, respectively. The concentrations of the natural hirudin, the homogenized gene-transformed mammary gland cell solution and the gene-transformed mammary gland cell culture medium needed for the anti-coagulation reaction at the A_{405} reading of 0.05 are 0.52 pmole, 0.056 mg and 0.95 μL, respectively.

Example 5: Determination of Expression Condition of Hirudin-expressing Vector in Mammary Gland Tissue

5.1: Handling of Mammary Gland Tissue

[0056] ICR female mice in lactation were sacrificed on the 11th day after delivery, and the mammary gland tissue thereof was taken. The *in vitro* mammary gland tissue was rinsed with 1-time PBS for 3 times and was centrifuged at a low speed of 1,000 rpm for 15 minutes in order to wash off the milk. The mammary gland tissue was cut into 8-mm^3 pieces. Weighed 0.25 g tissue and suspended in 0.8 mL DMEM medium.

5.2: Gene Transformation of Mammary Gland Cells

[0057] Gene transformation was carried out by the electroporation method. pE-αLA-Hi plasmid DNA (40 μg), was added into the mammary gland tissue obtained from Step 5.1. After the mammary gland tissue and DNA were uniformly mixed for 10 minutes, they were put into an electroporation cuvette with a width of 0.4 cm and treated by an electroporator (ECM 2001, BTX, USA) under the condition of 200 V/cm, 50 ms for 6 times. The treated mammary gland tissue was moved into a 35-mm petri dish and the medium was changed to DMEM medium containing 5 μg/mL insulin , 5 μg/mL prolactin, 1 μg/mL dexamethasone and 10% FBS.

[0058] The gene-transformed mammary gland tissue was cultured in an incubator at 37°C, 5% CO_2 for 48 hours, and anti-coagulation activity of hirudin in the culture was determined. The mammary gland tissue was then homogenized, and the anti-coagulation activity of hirudin in the homogenized solution was determined.

5.3: Determination of Anti-coagulation Biological Activity

[0059] Determination of anti-coagulation biological activity was carried out with various amounts (0.03, 0.06, 0.43, 0.25, 0.5, 1 and 2 mg total protein), of homogenized gene-transformed mammary gland tissue solution or various volumes (0.15, 0.31, 0.62, 1.25, 2.5, 5 and 10 μL), of gene-transformed mammary gland tissue culture medium as described in Step 4.2.

[0060] As shown in Fig. 6 and Fig. 7, when the concentration of the natural hirudin is 0.16 pmole, there is almost no coagulation; a similar no-coagulation situation happens when the amounts of the homogenized gene-transformed mammary gland cell solution and the gene-transformed mammary gland cell culture are 1 mg and 10 μL, respectively. The concentrations of the natural hirudin, the homogenized gene-transformed mammary gland cell solution and the gene-transformed mammary gland cell culture needed for the anti-coagulation reaction at the A_{405} reading value of 0.05 are 0.52 pmole, 0.11 mg and 1.88 μL, respectively.

Example 6: Transgenic Animals

6.1: Preparation of Transgene

[0061] The pE-αLA-Hi was digested with restriction enzymes, *Cla*I and *Dra*III, to yield a 4.77 kb DNA fragment which containing αLA promoter sequence, hirudin open reading frame and SV40 poly A tail sequence. The above DNA fragment (named αLA-hirudin) was electrophorized and recovered from 1% agarose gel by Gel Extraction Kit and diluted with

T.E. buffer (10 mM Tris-HCl, 0.25 mM EDTA, pH 7.4) to 1 ng/μl for murine and porcine pronuclear microinjection.

6.2: Generation of Transgenic Mice

[0062] Mature ICR mice were used as embryo donors and recipients. All mice are reared in a clearance laboratory rodent house, conditioned at 20°C to 26°C, ventilated by HEPA system, with 14 hours photo-period, fresh water and feed are supplied *ad libitium*. All donors were superovulated by i.p. injection of PMSG (pregnant mare serum gonadotropin , 10 IU, China Chem. and Pharm., Taiwan) and i.p. injection of human chorionic gonadotropin (hCG) (10 IU, purchased form China Chem. and Pharm., Taiwan), at 48 hrs post PMSG injection, and then were mated with stud ICR male at the day of injection hCG.

[0063] Fertilized zygotes were flushed from oviducts and the pronuclear embryos were micromanipulated by Narishige manipulator with differential interference contrast inverted microscope, (Nikon, Japan). The transgene was injected into the male pronucleus of mouse embryos. The injected and survived embryos were grouped about 25 to 30 and transferred into the fallopian tubes of foster dams, with copulatory plugs put into place after mating with vasectomized male, as soon as possible. After laboring, pups were nursed for three weeks and then a small piece of tissue was cut from the tail for genomic DNA extraction to screen the exogene by PCR.

6.3: Generation of Transgenic Pigs

[0064] Pure breed Landrace (L), Yorkshire (Y), Duroc (D) or their cross-bred (LY) gilts being at least seven and half months old were used. The animals were fed with 1.0 to 1.2 kg commercial feed twice daily and access to water *ad libitium*. Lactation sows were fed with lactation feed. The transgenic piglets were weaned at 28 days after delivery.

[0065] All embryo donor and recipient gilts were synchronized by feeding Regumate® (containing 0.4% altrenogest; 20 mg/day; Intervet, Boxmeer, Netherlands), mixed with commercial feed in the morning for 15 days, superovulated by injection of PMSG (1,500-2,000 IU, i.m., purchased from China Chem. and Pharm., Taiwan), at 24 hrs after the last feeding Regumate® and injection of hCG (1,250-1,750 IU), at 76-78 hrs after the injection of PMSG, and serviced by artificial insemination with pure breed L, Y, or D boars' fresh-diluted semen at 24-36 hrs after the injection of hCG.

[0066] At 54-56 hrs after hCG injection, the donor pigs were surgically operated on to flush

fertilized zygotes from the fallopian tubes with 20 ml Dulbecco's-PBS (purchased from Gibco/BRL, USA) with 0.4% BSA (purchased from Fraction V, Sigma, USA) into a dish. Before operation, pigs were fasted overnight, and were sedated by injection (i.m.) with 5 ml sterinil (2 mg/kg, purchased from Janssen Pharmaceutical, Belgium) and 10 ml atropine sulfate (90.04 mg/kg, purchased from China Chem. and Pharm., Taiwan). Then, they were initially anaesthetized by injection of sodium pentobarbitone (10 mg/kg, purchased from Abbott Australasia Pty Ltd., Australia), into an ear vein. Anesthesia was maintained throughout the operation by 4% halothene (purchased from ICI Ltd., USA), inhalation. Embryos were surgically transferred into the fallopian tube of other synchronized foster pigs with the same procedures as for donors. Upon the fallowing, a small piece of the piglet's ear or tail tissue was taken to extract their genomic DNA for analysis.

[0067] The fertilized zygotes were centrifuged with 23,500 ×g for 8 min. in room temperature by centrifuge (Hettich EBA 12, Germany), to expose pronuclei. The pig embryos were micromanipulated by Leica mechanical manipulator with differential interference contrast inverted microscope, (ZEISS Axiovert 135, Germany). The transgenes were injected into the pronucleus of new fertilized zygotes or nuclear of two-cell stage of pig embryos. After 25 to 30 pig embryos were injected, the embryos were transferred into the fallopian tubes of recipient - synchronized as soon as possible.

[0068] As showed in Table 1, there were 383 mice embryos and 180 pig embryos injected and transferred into 15 and 8 foster dams, respectively. After pregnancy, there were 30 mouse pups and 18 piglets born, of these 5 mice and 1 pig proved to be transgenic.

Table 1. Generation of αLA-hirudin transgenic mice and pigs by pronuclear microinjection

Animal	No. of embryos		No. of foster		No. of pups or piglets	
	Microinjection	E.T	Microinjection	Pregnant (%)	Born	Transgenic (%)
Mouse	563	383	15	10 (66.7)	30	5 (16.7)
Pig	180	180	8	4* (50.0)	18	1 (5.6)

E.T = embryo transfer

* One sow is still in pregnancy.

6.4: Analysis of Transgenes

[0069] Upon the delivering of mouse pups or piglets, the tail tissue of live mice or ear tissue

of piglets, respectively, were taken to extract genomic DNAs as PCR template at the weaning or delivery day.

[0070] The transgene was screened by PCR with following specific primer pairs which designated according to up strand of αLA promoter sequence and down strand of hirudin sequence.

[0071] (SEQ ID NO:11)Forward primer: 5'-GCT TCC TAG AAC CAA CAC TAC CAG-3'

[0072] (SEQ ID NO:12)Reverse primer: 5'-GTC GCC GTC GTT GTG AGA CTG -3'

[0073] Taking above template DNAs (1ng each) and pE-αLA-Hi (1ng, as positive control) to add into respective PCR reaction mixture which containing 10-time PCR buffer (10 μl, comprising 15 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100, 0.1% gelatin and 100 mM Tris-HCl, pH 7.9), dATP, dCTP, dTTP and dGTP (200 μM each), the above primer pair (0.2 μM each) and 0.5U *Tth* polymerase. The PCR mixtures were heated at 94°C for 3 minutes before entering the PCR cycles. The reaction conditions are 94°C for 60 seconds, 55°C for 60 seconds and 72°C for 60 seconds. After a total of 40 cycles, the mixtures were subjected to 72°C for 3 minutes to complete the DNA extension. Then the PCR products were electrophorized by 2% agarose gel and the PCR products are 472 bp in length as showed in Fig.8.